A cancer-associated PCNA expressed in breast cancer has implications as a potential biomarker

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Two isoforms of proliferating cell nuclear antigen (PCNA) have been observed in breast cancer cells. Commercially available antibodies to PCNA recognize both isoforms and, therefore, cannot differentiate between the PCNA isoforms in malignant and nonmalignant breast epithelial cells and tissues. We have developed a unique antibody that specifically detects a PCNA isoform (caPCNA) associated with breast cancer epithelial cells grown in culture and breast-tumor tissues. Immunostaining studies using this antibody suggest that the caPCNA isoform may be useful as a marker of breast cancer and that the caPCNA-specific antibody could potentially serve as a highly effective detector of malignancy. We also report here that the caPCNA isoform functions in breast cancer-cell DNA replication and interacts with DNA polymerase δ. Our studies indicate that the caPCNA isoform may be a previously uncharacterized detector of breast cancer.

mass spectrometry | pathology | posttranslational modification | DNA replication | genome stability

Despite advances in imaging assessment and treatment protocols and significant efforts in educating the public to the benefits of yearly breast examinations, >40,000 women die of metastatic breast cancer each year in the U.S. (1). Expression of proliferating cell nuclear antigen (PCNA) by cells during the S and G2 phases of the cell cycle makes the protein a good cell-proliferation marker (2, 3). In addition, immunohistochemical staining of PCNA has been used extensively in breast cancer diagnosis and prognosis (2, 4–6). PCNA has proven to be a useful marker to evaluate cell proliferation and prognosis when combined with other breast cancer markers, such as estrogen receptor, progesterone receptor, and Her2/neu (2, 7–9). Increased PCNA expression was also shown to be related to a shorter disease-free period and overall survival time in patients with breast cancer (5).

PCNA has been called the "ringmaster of the genome," because this 29-kDa protein has been shown to actively participate in a number of the molecular pathways responsible for the life and death of the mammalian cell (10). There are a number of reports in the literature suggesting that PCNA is, itself, posttranslationally modified, although there is some conflict as to what these modifications may be (11–14).

Previous data from our laboratory using 2D PAGE indicated that there were two PCNA isoforms in breast cancer cells, whereas only one isoform of PCNA was observed in nonmalignant breast cells (15). We determined that the cancer-associated isoform of PCNA (caPCNA) does not arise because of a genetic mutation but, more likely, as a result of posttranslational modification. In addition, we have shown that breast cancer cells carry out an error-prone DNA synthesis both *in vitro* and *in vivo* (16, 17). Therefore, additional studies to structurally and functionally understand the role that caPCNA plays in breast cancer cells are warranted. This information could potentially be exploited for both the detection and treatment of the disease.

Current commercially available antibodies recognizing PCNA interact with the single PCNA isoform found in nonmalignant breast cells as well as both isoforms observed in breast cancer cells. By using these antibodies, it is impossible to differentiate between the PCNA isoforms in malignant and nonmalignant breast cells and tissues by standard immunohistochemical staining procedures. We report here the successful development of an antibody that specifically detects the caPCNA isoform expressed by breast cancer cells and tumor tissues. A variety of immunostaining studies using this antibody suggest that the caPCNA isoform may be useful as a marker of breast cancer and that this antibody could serve as a highly effective detector of malignancy.

Results

Development of an Antibody That Specifically Identifies caPCNA. We had observed, using 2D PAGE, that nonmalignant breast epithelial cells contain a single isoform of the PCNA protein that has a basic isoelectric point (nmPCNA). Malignant breast epithelial cell cultures and breast epithelial cells in tissues, on the other hand, were found to harbor the basic form of the protein as well as an acidic isoform (caPCNA) (15). These results suggested that caPCNA has the potential to serve as an effective marker for identifying patients harboring malignant breast epithelial cells. To explore this possibility further, we recently developed an antibody against caPCNA. A rabbit polyclonal antibody (caPCNAab) was prepared against a peptide fragment of the PCNA protein. Two-dimensional PAGE Western blot analysis of a MCF7 breast cancer cell extract was performed to evaluate the antibody's ability to specifically recognize caPCNA by using either commercially available anti-PCNA PC10 antibody or our polyclonal caPCNAab antibody (Fig. 1*A*). PC10 antibody clearly bound both the nmPCNA and caPCNA isoforms, whereas caPCNAab recognized only the caPCNA isoform.

Comparative Western Blotting Analysis of a Panel of Breast Cancer and Normal Breast Tissue Specimens Using caPCNAab and Commercially Available Antibodies. A panel of normal breast tissue and breast cancer tissue specimens was analyzed by Western blotting for

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Abbreviations: DCIS, ductal carcinoma *in situ*; HMEC, human mammary epithelial cell; PCNA, proliferating cell nuclear antigen; SV40, simian virus 40.

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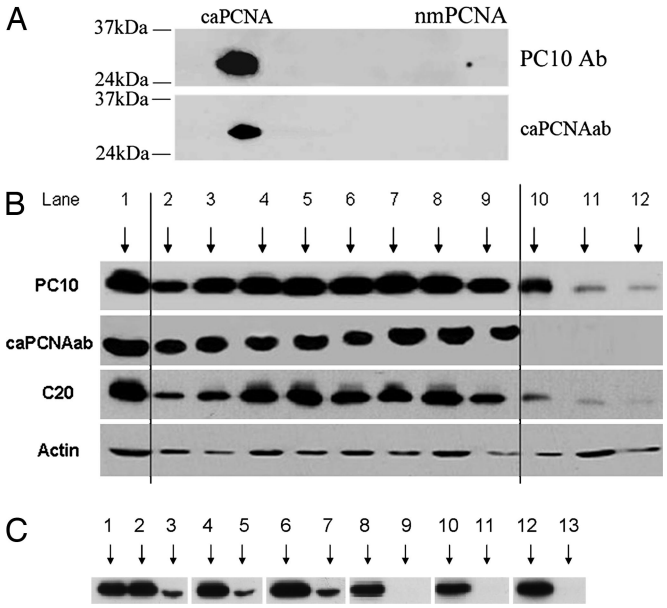

Fig. 1. Western blot analyses of tissue and cell line extracts. (*A*) caPCNAab antibody specifically recognizes the caPCNA isoform. Sixty micrograms of MCF7 cell extract were subjected to 2D PAGE and Western blot analysis using our procedures (15). The PC10 and caPCNAab antibodies were used at a dilution of 1:1,000 in the Western blot analysis. (*B*) caPCNAab recognizes the form of PCNA uniquely expressed in malignant tissues. Two-hundred-microgram aliquots of each tissue extract were subjected to 1D PAGE and Western blot analysis using our procedures (15). The PC10, C20, and caPCNAab antibodies were used at a dilution of 1:1,000 in the Western blot analysis. Detection of actin protein by actin antibody, at a dilution of 1:1,000, was used as a loading control for the extracts in the experiments. Lane 1, MCF-7 cell extract (serves as a marker for PCNA); lanes 2–9, extracts representing cancer tissue from eight different women with breast cancer; lanes 10–12, normal breast tissue extracts prepared from three different women. (*C*) High concentrations of caPCNAab in Western blot analysis does not recognize the PCNA isoform present in nonmalignant breast tissues. Two hundred micrograms of tissue extracts, prepared from either a woman with breast cancer or a woman who was disease free, were subjected to 1D PAGE and Western blot analysis using our procedures (15). The PC10 and caPCNAab antibodies were used at dilutions of 1:250, 1:500, or 1:1,000 in the Western blot analysis. Lane 1, MCF7 cell extract (serves as a marker for PCNA); lanes 2, 4, and 6, breast cancer tissue extract probed by using PC10 antibody at a dilution of 1:1,000, 1:500, or 1:250, respectively; lanes 3, 5, and 7, nonmalignant breast tissue extract probed by using PC10 antibody at a dilution of 1:1,000, 1:500, or 1:250, respectively; lanes 8, 10, and 12, breast cancer tissue extract probed by using caPCNAab at a dilution of 1:1,000, 1:500, or 1:250, respectively; lanes 9, 11, and 13, nonmalignant breast tissue extract probed by using caPCNAab at a dilution of 1:1,000, 1:500, or 1:250, respectively.

the presence of PCNA using either commercially available antibodies or caPCNAab (Fig. 1*B*). The commercially available antibodies included C20, an antibody to the C terminus of PCNA, and PC10, prepared against a full-length rat PCNA protein molecule. It was observed that the commercially available antibodies readily recognized the PCNA present in either the normal or malignant breast tissue extracts. However, the caPCNAab antibody detected the presence of PCNA only in malignant tissue extracts. The results shown here were from films that were developed overnight to determine whether any caPCNAab signal could be detected in normal breast tissue extracts. Presumably, this unique ability of caPCNAab to detect PCNA only in breast cancer cell extracts is because of the acidic isoform of PCNA being expressed in the malignant cells and not in normal cells.

The specificity of the antibody for the caPCNA isoform was further demonstrated in an experiment in which increasing concentrations of either the PC10 antibody or caPCNAab were used in Western blot analyses of nonmalignant and malignant tissue extracts for PCNA detection (Fig. 1*C*). The results of this experiment clearly show that, even at high concentrations of the caPCNAab in the Western analysis, the antibody detected the presence of PCNA only in cancer tissue, whereas, at any concentration, the PC10 antibody readily detected PCNA protein in both malignant and nonmalignant breast tissue.

caPCNAab Specifically Recognizes Breast Cancer Epithelial Cells Grown in Culture and Present in Human Tissue. Immunofluorescence analyses were performed to evaluate whether caPCNAab could distinguish between malignant and nonmalignant breast epithelial cells (Fig. 2 *A–C*). The results demonstrate that the antibody has specificity for breast cancer cells grown in culture (Fig. 2*A*). Normal human mammary epithelial cells (HMECs), nonmalignant spontaneously immortalized HMECs, transformed HMECs (see *Methods*), and MCF 7 breast cancer cells were used. All of these different cell types were stained with DAPI (blue) to demonstrate the presence of cells in each magnification field. As can be seen in Fig. 2*A*, the PC10 antibody (in green) readily stained each of the different cell types examined, both malignant and nonmalignant. However, unlike the PC10 antibody, the caPCNAab antibody (in red), does not stain nonmalignant epithelial cells but is able to readily detect breast cancer cells. DAPI staining (blue) of the nonmalignant epithelial cells, which stains the nucleus of these cells, does show the presence of cells in the field. The few bright red fluorescent "spots" seen in the nonmalignant cultures stained with caPCNAab appear to be due to nonspecific binding to debris, because these spots are also seen in the same location in the cultures stained with the green-labeled PC10 antibody but not DAPI. This study demonstrates that caPCNAab can specifically detect cancer cells grown in culture.

In a related study, paraffin-embedded nonmalignant and malignant breast tissue specimens were also evaluated by comparative immunofluorescence staining using commercially available antibodies and caPCNAab (Fig. 2*B*). In this study, the commercially available PC10, C20, and 100-478 (Novus Biologicals, Littleton, CO) antibodies were evaluated. The 100-478 antibody was specifically prepared against the interconnector domain of the PCNA molecule. As can be seen in Fig. 2*B*, all of the commercially available antibodies (green) readily stain both nonmalignant and malignant breast tissue. In contrast, caPCNAab (red) stained only malignant breast tissue. These studies using both cells grown in culture and human tissue demonstrate that caPCNAab can detect breast cancer cells specifically and supports our premise that caPCNA may be a marker for malignancy.

caPCNAab Is Effective for the Immunohistochemical Staining of Malignant and Nonmalignant Paraffin-Embedded Breast Tissue. Immunohistochemical staining of paraffin-embedded breast tissue specimens was performed with caPCNAab. The tissues examined were nonmalignant tissue obtained after breast-reduction surgery as well as tissues from patients with atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS), and invasive or metastatic disease. Representative results are shown in Fig. 2*C*. Clear and significant staining by the caPCNAab was observed only in the tissues from patients with DCIS, invasive, or metastatic disease. Tissues from disease-free women or those with ADH did not stain. These results also indicate that the caPCNAab specifically recognizes an epitope within the nuclei of cancer cells; cytoplasmic staining was not generally seen.

caPCNA Expression in Nonmalignant and Malignant Breast Tissues. Table 1 summarizes the results of a more extensive immunohistochemical analysis of breast tissue specimens using the caPCNAab and PC-10 antibodies. Expression of caPCNA was analyzed in normal lobules of breast tissue obtained from patients undergoing reduction mammoplasty for macromastia. In the 10 specimens

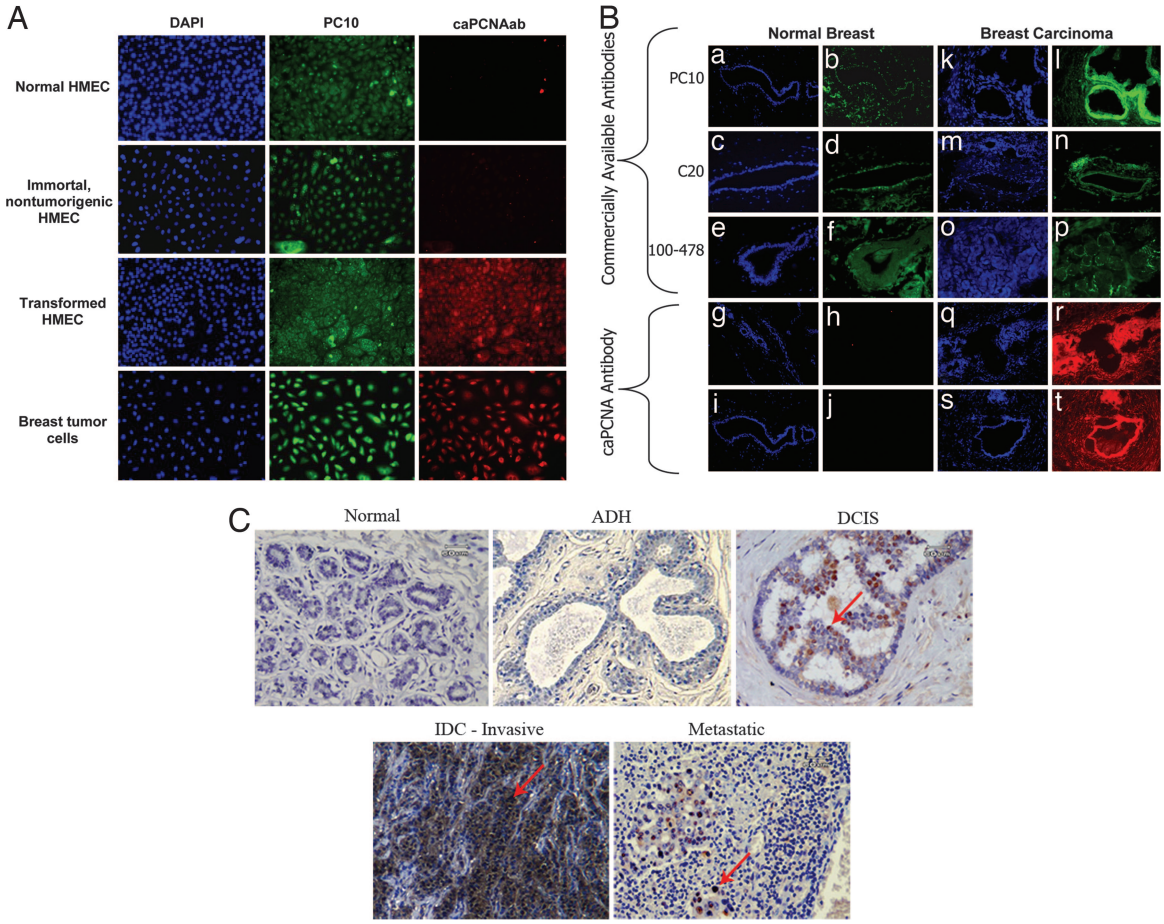

Fig. 2. Immunohistochemistry of cells grown in culture or tissue sections. (*A*) Tumorigenic breast epithelial cells express caPCNA, whereas nontumorigenic breast epithelial cells do not. See *Results* for a description of the experiment. These results are representative of two different experiments. The magnification used was ×200. (*B*) caPCNAab antibody specifically recognizes breast carcinoma cells and not normal cells in paraffin-embedded tissue. Normal breast tissues (3 μm) were obtained from three different patients: (*i*) *a* and *b* and *e* and *f*, (*ii*) *c* and *d* and *g* and *h*, and (*iii*) *i* and *j*. Breast carcinoma tissue sections (3 μm) were from three different patients: (*i*) *k* and *l*, (*ii*) *m* and *n* and *q* and *r*, and (*iii*) *o* and *p* and *s* and *t*). *a*, *c*, *e*, *g*, *i*, *k*, *m*, *o*, *q*, and *s* were stained with DAPI. These results are representative of two different experiments. The pictures were taken at a magnification of ×200. (*C*) Immunohistochemical staining using caPCNAab antibody can detect breast cancer cells in tissue. Results presented are representative of normal breast tissue derived after breast reduction surgery, breast tissue derived from a patient with atypical ductal hyperplasia, breast tissue derived from a patient with DCIS, tissue derived from a patient with invasive breast cancer, and tissue from a woman with metastatic disease. Slides were stained as described in *Methods*. Arrows indicate caPCNAab antibody staining of malignant breast cells.

analyzed, the vast majority of the lobules did not show any expression of caPCNA. However, in rare, often distorted lobules, some light expression of caPCNA in the nuclei was noted. These cells were often unusual in shape, relative to their neighbors. Epithelial cells in normal lobules from patients with cancer showed a staining intensity and pattern that resembled that of the distorted lobules. In addition, in these patients, the lobules immediately adjacent to the tumor sometimes exhibited nuclear expression in up to 2–3% of cells. However, there was a very significant increase in the frequency of caPCNA expression and the intensity of nuclear staining in specific foci of DCIS specimens. The frequency of caPCNA expression in DCIS and in invasive tumors, although variable from lesion to lesion, was usually >5% and averaged 30%. caPCNA was expressed in the nuclei of all the tumor cells in all the cases of breast cancer examined (consisting of invasive ductal carcinoma, metastatic breast cancer, and pure DCIS specimens). The histologic grade of the tumor did not influence the percentage of cells or intensity of staining with caPCNAab. In addition to nuclear expression, reactivity of caPCNAab with caPCNA in the cytoplasm was noted in some tumors. In contrast to the staining results obtained with caPCNAab, the staining of serial sections with the PC-10 antibody resulted in a higher percentage of cells staining with the PC-10 than with the caPCNA-specific antibody (data not shown).

Table 1. Immunohistochemical analysis of malignant and nonmalignant breast tissue

		caPCNA expression		
Tissue type	No. of cases	Average staining intensity	Average percentage of cells stained	Total score (intensity × %)
Normal (reduction mammoplasty)	10	2	0.5	1
Normal (cancer patients)	35	2	2	4
DCIS	30	3	30	90
Invasive carcinoma	55	3	40	120

caPCNA Actively Participates in Breast Cancer Cell DNA Replication and Can Interact with the Cell's DNA Polymerase δ. PCNA functions as a DNA polymerase δ accessory factor in mammalian cells (18,

Table 2. Effect of caPCNAab on DNA replication and DNA polymerase δ activities

	Percent inhibition	
caPCNab concentration, μg	*In vitro* SV40 DNA replication*	DNA Pol δ†
0.1	29	N/D
2.0	40	34
5.0	N/D	57
10.0	48	63

Prior to reaction initiation, the MCF7 protein fraction was incubated with increasing concentrations of caPCNAab for 1 h at 4°C. Data represent the mean of three independent experiments. N/D, not determined.

*The SV40 *in vitro* DNA replication assay was performed according to our procedures (21–29).

†The DNA polymerase δ assay was performed according to the procedures of Han *et al.* (35).

19). PCNA has also been identified as an essential factor for simian virus 40 (SV40) DNA replication *in vitro* (20). To determine whether caPCNA actually plays a role in breast cancer cell DNA replication and functions with DNA polymerase δ, we performed DNA polymerase δ and *in vitro* SV40 DNA replication assays in the absence and presence of increasing amounts of caPCNAab, using a MCF7 cell extract (Table 2). It was observed that caPCNAab inhibited both DNA polymerase δ and *in vitro* SV40 DNA replication activity in the breast cancer cell extract. BSA was added to the control reactions, and no inhibition was noted (data not shown).

Discussion

Much active research is currently being directed at discovering those molecular signatures that can serve as markers (biomarkers) for detection and potentially be of aid in the treatment of cancer, because the greatest potential for reducing mortality associated with breast cancer lies in both the detection of asymptomatic, early-stage disease and the presence of residual disease after treatment. Thus, the discovery of markers for these purposes holds significant promise for combating the disease. Additionally, a biomarker with both significant sensitivity and specificity would help to clarify ambiguities related to making the correct diagnosis and, consequently, selecting the appropriate course of treatment for patients lacking a frank malignancy.

One of the critical regulatory points controlling human cell proliferation occurs at the level of DNA replication. Our laboratory successfully isolated, extensively purified, and characterized an intact mammalian cell multiprotein DNA-replication complex from a variety of human cell types that was both stable and fully functional (21–29). We proposed a model to represent the mammalian multiprotein DNA replication complex or DNA synthesome (22, 23). We have also observed that malignant breast epithelial cells replicate DNA with a lower fidelity than their nonmalignant-cell counterparts. This observation suggested that this characteristic may be a hallmark of breast malignancy. We also observed that the reduction in DNA synthetic fidelity correlated with the appearance of a unique isoform of a component of the synthesome (caPCNA) (15). caPCNA expression appeared to be associated with malignant breast cells and correlated with the reduction in DNA synthetic fidelity exhibited by these cells.

In this report, we showed the results of Western blot analyses of malignant and nonmalignant breast epithelial cell line and tissue extracts resolved by SDS/PAGE and probed with caPCNAab. These data indicated that the antibody selectively identified the caPCNA isoform expressed by the cancer cells. The apparent selectivity of caPCNAab for the caPCNA isoform and its failure to detect the nmPCNA isoform expressed by nonmalignant breast epithelial cells could have arisen from insufficient amounts of caPCNAab in the Western blot reaction mixture. This possibility was tested in two ways. First, we examined Western blots of extracts of both malignant and nonmalignant breast tissue specimens resolved by 1D PAGE using equivalent amounts of PC10 and caPCNAab. Our results indicated that only the PC10 antibody could detect PCNA in the nonmalignant breast tissue specimens (Fig. 1*B*). We then determined whether increasing the concentration of caPCNAab in the Western blot incubation mixture would enable us to identify the presence of PCNA in the nonmalignant breast specimens (Fig. 1*C*). Again, our results indicated that, despite a fourfold increase in caPCNAab concentration over the initial amount used in Fig. 1 *A* and *B* and an equivalent signal intensity for both the PC10 and caPCNAab for the PCNA present in malignant breast tissue extracts resolved by 1D PAGE (lanes 6 and 12), no PCNA band was observed in the nonmalignant specimen probed with caPCNAab (lane 13), whereas the PCNA expressed in the nonmalignant specimen resolved in lane 7 was readily detected by the PC10 antibody.

Immunohistochemical staining of breast tissue specimens using caPCNAab (Table 1) indicated a negligible level of staining of paraffin-embedded normal breast tissue specimens obtained from patients either undergoing reduction mammoplasty or who remain disease-free after treatment for invasive breast cancer. Histologically normal breast tissue taken from areas adjacent to breast tumors showed a slightly elevated frequency of caPCNA expression in these adjacent normal cells. Histologically normal tissue specimens taken from reduction-mammoplasty patients demonstrated a negligible frequency and intensity of staining with caPCNAab. In contrast, every histologically distinct malignant breast cell studied expressed caPCNA and stained intensely with caPCNAab. The grade of the tumor specimen did not appear to influence either the frequency or level of intensity of staining with caPCNAab. These data were consistent with the immunofluorescence data of malignant and nonmalignant breast tissue specimens shown in Fig. 2.

The true "why" the caPCNAab antibody recognizes caPCNA specifically, as opposed to the commercially available monoclonal antibodies recognizing PCNA, will not be clear until a full structural analysis of nmPCNA is completed. We have very recently described the first mass spectral analysis for any isoform of PCNA (30) and detailed the posttranslational modifications present on the caPCNA isoform. A comparison of the caPCNA posttranslational modifications with that of nmPCNA should give us insights into not only the altered posttranslational modification pathway(s) responsible for the expression of these different isoforms in breast cancer cells but also the sites of modification on the protein that may differ between the two isoforms. But, at this point, we can only propose that a posttranslational modification is present in the nmPCNA isoform that is absent in caPCNA and that it somehow physically blocks the ability of the antibody to recognize the nmPCNA isoform. Alternatively, the posttranslational modification on nmPCNA results in an altered conformation from that exhibited by caPCNA; and this altered conformation in nmPCNA may make it unrecognizable to the caPCNAab antibody, even in denaturing polyacrylamide gels. It has long been known that PCNA resolves anomalously in SDS polyacrylamide gels. The molecular weight of mammalian PCNA, estimated by SDS/PAGE, differs notably from that predicted by the cDNA sequences, that is, 36,000 in comparison with 29,261 and 28,748 for human and rat PCNA, respectively. Studies published over the years suggested that the size discrepancy may be due to the protein sequence *per se*, namely a sequence-related anomaly in SDS/PAGE (31–33). Conceivably, the observed and long-time known anomalous behavior of PCNA in SDS gels, together with a potential conformation change in nmPCNA (induced by posttranslational modification), could be imagined to make the nmPCNA isoform "invisible" to the caPCNA-specific antibody. However, this is purely speculation until a nmPCNA isoform mass spectral analysis is completed.

We also demonstrated in this report that caPCNA interacts with both DNA polymerase δ and functions in breast cancer cell DNA

replication, because caPCNAab inhibits both *in vitro* DNA replication and DNA polymerase δ activities when added to the appropriate reaction mixtures (Table 2). In contrast, an equivalent amount of PBS added to the replication and DNA polymerase δ reaction mixtures does not inhibit either activity. An implication of these findings is that caPCNA may play an active role in the error-prone DNA synthesis observed in these breast cancer cells.

We recently reported on the mass spectral analysis of the caPCNA isoform (30). This analysis did not identify the presence of acetylated, ribosylated, or phosphorylated forms of PCNA. Both our 2D PAGE and mass spectral analyses ruled out the possibility that the two isoforms differ in electrophoretic mobility because of differences in structure resulting from ubiquitination and/or sumolyation of the PCNA polypeptide. Our mass spectral analysis of the caPCNA isoform did indicate the presence of an unusual form of methylation on both specific glutamate and/or aspartic acid residues within the PCNA polypeptide and suggested that methyl esterification of acidic amino acids may be associated with alterations in the function of PCNA. We therefore speculate that aberrant methylation of key proteins could play a role in mediating the reduction in DNA synthetic fidelity, reduced DNA repair efficacy, and abrogation of key cell-cycle check points leading to the transformation of normal cells and their progression into highly metastatic tumors.

We also observed, in preliminary studies using 2D PAGE that, in addition to being expressed in breast cancer cells and tissues (15), caPCNA is present in the sera of untreated breast cancer patients (L.H.M. and R.J.H., unpublished data). These data point to the potential of caPCNA to serve as a marker for identifying patients harboring malignant breast cells and for monitoring remission status. To explore this possibility further, we developed an antibody (caPCNAab) and demonstrate, in this report, that caPCNAab specifically recognizes caPCNA expressed by breast cancer cells in malignant breast tissue specimens or breast epithelial cancer cells grown in culture. Taken together, these studies suggest that the caPCNA isoform may be a marker of breast epithelial cell malignancy. The translational implications of this work are (*i*) the caPCNAab antibody may be useful for monitoring the remission status of individuals being treated for breast cancer, (*ii*) caPCNAab may be a useful reagent for developing ELISA and immunohistochemical assays for screening purposes, (*iii*) the degree of caPCNAab expression may be useful in identifying patients at high risk of metastasis or relapse to facilitate adjuvant treatment decisions, (*iv*) caPCNAab may become a useful member of a panel of antibodies that have the ability to recognize high-risk lesions for their malignant potential, and (*v*) caPCNAab may be linked to imaging modalities to evaluate the presence of primary or metastatic tumors. Additional examination of the caPCNA antibody will determine its potential usefulness in cancer detection, risk assessment, and prognosis.

To date, all of our data point to caPCNA as having an important role in the life of a breast cancer cell. It has always been found where there is a diagnosis of malignancy, and its presence correlates with a decrease in DNA-replication fidelity. This evidence indicates that a thorough structure and function analysis of caPCNA in breast cancer cells may lead to important insights into its role in cancer-cell proliferation and progression. Because PCNA interacts with a wide variety of binding partners, our discovery of caPCNA in breast cancer cells (and its participation in breast cancer cell DNA replication and interaction with DNA polymerase δ), suggests a mechanism whereby specific posttranslational modifications play a key role in abrogating cell-cycle checkpoints that depend on PCNA function. Expression of caPCNA may also contribute to the cascade of events that lead to the accumulation of genetic damage sustained by the breast cancer cell and, ultimately, to the transformation of the normal breast epithelial cell. In addition, because PCNA is an essential component of both the DNA replication and repair machinery, caPCNA expression in malignant breast cells is likely to have a role in mediating the reduced fidelity with which the cancer cell maintains its genome. We believe that our discovery of caPCNA will enable the eventual elucidation of a heretofore unrecognized molecular mechanism contributing to the reduction in DNA replicative fidelity exhibited by the breast cancer cell.

Since the submission of this manuscript, we have performed a limited number of studies to address whether caPCNA was expressed in epithelial cells of cancers derived from other organ types. In these studies, we have observed that caPCNA is, indeed, expressed in other cancer types. We have tested esophageal cancer, colon cancer, neuroblastoma, and ovarian cancer and have observed caPCNA expressed only in the malignant tissues but not to any significant extent in their nonmalignant counterparts.

Methods

caPCNAab Preparation. Rabbit polyclonal antibodies were prepared by a commercial vendor, (Zymed, San Francisco, CA), to a synthesized peptide fragment of PCNA coupled to keyhole limpet hemacyanin (KLH) through four cysteines residues added to the amino-terminal portion of the peptide. One hundred micrograms of the KLH conjugated to a peptide fragment of PCNA contained within amino acids 123–140 of the protein was resuspended in complete Freund's adjuvant, and injected s.c. into multiple sites in two female New Zealand White rabbits. The rabbits were rested for one month before boosting the animals with a second 100-μg dose of the KLH-coupled antigen in incomplete adjuvant. The antibody titer to the antigen was determined by ELISA ≈10–14 days after immunization, and, after an additional 14-day rest period, the animals received another boost of KLH-coupled antigen. Twelve days later, 25 ml of antisera was collected from each rabbit and stored at −20°C. The antisera were dialyzed against two changes of 20 mM PBS, pH 7.0, and loaded onto a protein G Sepharose column preequilibrated with the PBS. The binding capacity of the gel was 19 mg of rabbit IgG per ml of packed gel bed. The column was washed with 10 column volumes of PBS and eluted with 10 volumes of 0.1 M glycine buffer, pH 3.0. One-milliliter fractions eluting from the column were collected at a flow rate of 1–2 ml/per min into 0.25 ml of 0.25M Tris·HCl, pH 8.0. The concentration of protein in fractions containing the protein peak eluting from the column was determined by Bradford assay, and these fractions were combined and dialyzed against PBS containing 10 mM NaN_3 before being stored at 4°C until used in various analyses.

Sample Preparation, PAGE, and Western Blot Analysis. Normal breast tissue and breast cancer tissue specimens were cut into small pieces and frozen in liquid nitrogen. The tissue specimens were then crushed and ground into a powder by using a mortar and pestle. T-per (Pierce, Rockford, IL) (roughly 20 ml/g of tissue) was added to the tissues, and the tissues were then Dounce homogenized on ice by using a loose-fitting pestle. The homogenates were centrifuged at 10,000 × *g* for 10 min. The protein concentrations of the supernatants were measured by using the Bradford method. Protein aliquots from each homogenized tissue specimen were resolved by electrophoresis in a 12% PAGE/SDS gel. The resolved proteins were transferred to a PVDF membrane, and the membrane was blocked with TNE blocking buffer (10 mM Tris·HCl, pH 7.5, 2.5 mM EDTA, pH 8.0, and 50 mM NaCl) with 5% fat-free milk and 1% Tween 20 for 1 h. Unless stated otherwise, a 1:1,000 dilution of the commercially available antibodies was used to detect PCNA, and the dilution of the appropriate secondary antibodies for the analysis was also 1:1,000. Unless stated otherwise, a 1:1,000 dilution of the caPCNAab antibody was used in the analysis with a 1:1,000 dilution of the appropriate secondary antibody. The dilution of primary and secondary antibodies to detect actin were each 1:1,000.

Commercially Available PCNA Antibodies. The commercially available antibodies used in these studies are as follows. PC10 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

and is a mouse monoclonal raised against rat PCNA made in the protein A expression vector pR1T2T. 100-478 antibody was purchased from Novus Biologicals and is a monoclonal antibody prepared against the PCNA interdomain connector loop, residues 107–196. C20 antibody was purchased from Santa Cruz Biotechnology and is a goat polyclonal affinity-purified antibody raised against a peptide mapping at the C terminus of human PCNA.

Immunofluorescence Analysis of Human Cell Lines. The HMECs used for these experiments were grown under serum-free conditions as described (34–36). To obtain the nontumorigenic, yet immortalized, cell line, HMECs were derived from a 31-year-old Li–Fraumeni Syndrome patient's noncancerous breast tissue (containing a germ-line mutation at codon 133 in one of the two alleles of the p53 gene [Met to Thr (M133T)] that affects wild-type p53 protein conformation). These cells undergo crisis around population doubling level 50–60 and spontaneously immortalize with a frequency of five in 10 million (36). A transformed HMEC line was established by infecting the preimmortal HMECs with hTERT (37) and H-RasV12 (38) and then collecting clones that grew in soft agar and nude-mice xenografts. MCF-7 breast carcinoma cells were grown in DMEM (Invitrogen, Carlsbad, CA) containing 10% cosmic calf serum (HyClone, Logan, UT) and 50 μg/ml gentamicin (Invitrogen). Cells were subjected to immunofluorescence staining with either mouse anti-PC10 (recognizing all forms of PCNA) or rabbit caPCNAab (recognizing caPCNA). Cells grown on coverslips overnight were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 before blocking with 3% BSA. Staining was performed with the PCNA antibodies diluted in PBS with 0.5% sodium azide and an Alexa Fluor-468 anti-mouse IgG or Alexa Fluor-568 anti-rabbit IgG-conjugated secondary antibody (Molecular Probes, Eugene, OR). The coverslips were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA), and cells were examined by using a Leica (Bannockburn, IL) fluorescent microscope. Cells were counterstained with DAPI and viewed with a Leica fluorescent microscope using a 20× objective.

Immunofluorescent Analyses of Human Tissues. Paraffin-embedded tissues cut into 3-μm sections were placed on glass slides and incubated in xylene twice for 10 min each to remove the paraffin. Slides were rehydrated with a series of ethanol washes (100–90-80–70-0% in distilled H_20) for 10 min each. Antigen retrieval was performed by using the Antigen Unmasking Solution (Vector Laboratories) according to instructions. Slides were placed in blocking buffer (3% BSA in PBS) for 30–60 min at room temperature. Mouse anti-PC10 (recognizing all forms of PCNA), anti-C20, Novus 100-478, or rabbit caPCNAab (recognizing caPCNA) at 1:200 dilution in blocking buffer were placed directly onto the tissue, covered with parafilm, and incubated in a humid chamber for 60 min at room temperature. After three 5-min washes in PBS, the slides were incubated with the appropriate fluorescent secondary antibody (Alexa Fluor-468 anti-mouse IgG or Alexa Fluor-568 anti-rabbit IgG; Molecular Probes) at a 1:600 dilution in blocking buffer, covered with parafilm, and placed in a humidified chamber for 30–60 min at room temperature in the dark. Another series of three 5-min washes was performed in PBS, and the slides were mounted with Vectashield containing DAPI. Tissue sections were examined by using a Leica fluorescent microscope with a 20× objective. DAPI served as a counterstain.

Immunohistochemical Staining of Paraffin-Embedded Breast Tissue Specimens. After institutional review board approval, cases of breast cancer were selected, as were cases of DCIS and atypical ductal hyperplasia. In addition, cases that showed normal breast tissue or benign fibrocystic changes were also selected. These patients did not have a current or prior diagnosis of breast cancer. Four-micron paraffin sections fixed to charged slides were deparaffinized in xylene (three changes) and hydrated with graded alcohols and distilled water. Antigen retrieval was performed in citrate buffer (pH 6.0) by using a microwave oven for 10 min and subsequent cooling for 20 min, followed by blocking of endogenous peroxidase activity with Peroxo-block (Zymed), and, after rinsing the slides in PBS, the slides were incubated with caPCNAab (dilution: 1:400) for 1 hour. The antigen–antibody reaction was visualized by the avidin–biotin-peroxidase (Zymed Picture Plus kit: HRP/Fab polymer conjugate) with diaminobenzidine (DABplus; DAKO, Carpenteria, CA) as the chromogen. These slides were counterstained with hematoxylin (Vector Laboratories) and then cleared in alcohol and xylene. The slides were mounted with Histomount (Zymed) and a coverslip and visualized. Substitution of primary antibody by PBS or isotype control antibody was done for negative controls.

***In Vitro* SV40 DNA Replication and DNA Polymerase δ Assays.** The *in vitro* SV40 DNA replication assay was performed as described in Malkas *et al.* (21). The DNA polymerase δ assay was performed as described in Han *et al.* (39).

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